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13. ABSTRACT ***(Maximum 200 Words)***

The main purpose of the conceptual grant is to provide seed money to explore an idea that may generate preliminary data for a research proposal of a more traditional grant. Our studies far exceeded this goal. As planned in the proposal, the research by the one-year funding focused on determining whether MEKK1-p38/JNK activity is increased in tamoxifen-resistant breast cancer cells. Our studies generated positive data that showed that both JNK and p38 activity is increased in breast cancer cells that become tamoxifen resistant. We also obtained data showing that the activation of the estrogen receptor by MEKK1 changed during the development of tamoxifen resistance of MCF-7 breast cancer cells. Our studies have resulted in one publication in a first class journal, an idea grant proposal submitted to FY02 DOD Breast Cancer Program and an abstract submitted to the Era of Hope meeting sponsored by DOD Breast Cancer Program.

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Table of Contents

INTRODUCTION

Background: Tamoxifen (TAM) treatment is the preferred first line therapy for estrogen receptor-positive breast cancers. Because of its clinical efficacy and low toxicity, there are 2 to 3 million women receiving TAM treatment in more than 70 countries. Unfortunately, not all patients respond to TAM and even those responsive ones acquire resistance during the treatment. The mechanism underlying the resistance of breast cancer cells to TAM is not clear. However, it is known that many TAM resistant breast cancers are still ER positive (1) and MCF-7 tumors which become resistant to TAM use TAM as a mitogenic stimulus (2). These suggest that the TAM resistant breast cancer cells may have gained some of the characteristics of uterine cells in which TAM lacks antagonistic activity and is known to behaves as an agonist. In a separate study with uterine cells, we found that, in endometrial cancer cells, the expression of an active MEKK1, a MAP kinase kinase kinase which activated both the Jun N-terminal Kinase (JNK) and p38, blocked the antagonistic activity of TAM and switched TAM to a full agonist.

On one hand, increasing evidence now suggests that growth factors and their receptors are involved in TAM resistance (2). On the other hand, MEKK1 was shown to be activated by multiple membrane signals including growth factors such as EGF (3) and the MEKK1-mediated activation of JNK and p38 is essential for the transformation by multiple oncogenes including Abl, Met, Src as well as mutant EGF receptors. Therefore, we propose that the acquired resistance of breast cancer cells to TAM during the treatment is due to the increased activity or expression level of MEKK1 and its downstream kinases and that the increased activity of both JNK and p38 together, like what we showed in uterine cells, switches TAM to an agonist, resulting in the resistance phenotype.

The studies proposed in the conceptual grant is to test the above hypothesis using MCF-7 as a model system.

BODY

As planned in the proposal, our studies in the funding period determined whether the change of MCF-7 from TAM-sensitive to TAM-resistant status by stepwise selection with increased concentrations of TAM is accompanied by increased activity of JNK and p38. We successfully established TAM-resistant MCF-7 cells and showed that the resistant cells contained higher amount of p38 and JNK activity. The protein level of both kinases was not changed. The data has been described in detail in a new grant proposal, an abstract and a paper. Please look at the materials in the appendix for a detailed description about the specific data.

KEY RESEARCH ACCOMPLISHMENTS

1. We have established a TAM-resistant cell line;
2. We have shown that the resistant cells express similar level of the ER as the parental TAM-sensitive MCF-7 cells;
3. We have determined the JNK activity and showed that the JNK activity, not protein level, is increased in the TAM-resistant cells;
4. We have determined the p38 activity and showed that the p38 activity, not protein level, is increased in the TAM-resistant cells.

REPORTABLE OUTCOMES

1. An Idea grant proposal to the FY02 Breast Cancer Program (**Appendix #1**)
2 Abstract to the FY02 Era of Hope meeting sponsored by DOD Breast Cancer Program (**Appendix #2**);
3. A paper published in Molecular and Cellular Biology (**Appendix #3**).

CONCLUSIONS

In conclusion, our studies in the funding period have followed the plan of the original proposal very well and positive data has been obtained which supported the original hypothesis that the increased p38 and JNK kinases contributes to the development of breast cancer's resistance to

tamoxifen treatment.

"**So What section**": The research may lead to the development of p38 and JNK inhibitors as a therapeutic agent for the reversal of the breast cancer cell's resistance to TAM.

Bibliography of all publications and meeting abstracts

1. Abstract to the FY02 Era of Hope meeting sponsored by DOD Breast Cancer Program (**Appendix #1**);
2. A paper published in Molecular and Cellular Biology (**Appendix #2**);

List of personnel (not salaries) receiving pay from the research effort

None

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I. BACKGROUND

A. General Introduction. One of the most outstanding features of breast cancer (B-Ca) is its sensitivity to estrogens (E2) and Selective Estrogen Receptor (ER) Modulators (SERMs). The effect of E2 and SERMs is mediated through the ER, ERα (1) and ERβ (2-4). Both ERs belong to the nuclear hormone receptor superfamily (5, 6), a group of ligand-regulated transcription factors that govern gene expression by directly binding DNA response elements or through the tethering to other sequence specific transcription factors. While the role of ERβ in breast cancer remains largely unknown, ERα has a well-established function in B-Ca cell growth and will be referred as the ER in the rest of the text without further distinction.

Among all endocrine manipulations, therapy with tamoxifen(TAM), the prototype of all SERMs, represent a major accomplishment for cancer chemotherapy. TAM provides a major advantage for women with B-Ca and, because of its proven efficacy and low toxicity, it was estimated that approximately 2-3 million worldwide receive this treatment. Besides treatment, TAM and other SERMs are being actively pursued as a chemopreventive agent for B-Ca development in healthy women who are at high risk.

Unfortunately, not all patients respond to TAM and even those responsive ones acquire resistance during the treatment. The exact mechanism underlying TAM resistance, either primary or acquired, remains largely unclear. However, it is known that most TAM-resistant B-Ca remains ER-positive (7). It was reported that women who had tumor recurrence while on TAM therapy could have tumor regression when TAM is withdrawn (8-11). MCF-7 tumors which become resistant use TAM as a mitogenic stimulus (12). These suggest that TAM-resistant B-Ca cells may have gained some of the characteristics of endometrial cancer (E-Ca) cells in which TAM lacks antiestrogenic activity and is known to be a growth stimulus.

Crystal structures of the ER bound to different ligands reveal that ligands of different size induce a spectrum of conformational states that are then interpreted by the cell type specific complexes of coregulators and the environment of the local promoter of the target genes. It is believed that receptor conformation, promoter context and coregulator complexes work together to mediate the tissue-specific effect of SERMs (13). Using chromatin immunoprecipitation(CHIP) assays, Shang and Brown (14) have recently shown that, while TAM-bound ER recruits corepressors (Co-R) to the promoter of ER target genes regulated through estrogen response elements (ERE) in both cells, it selectively recruits Co-R in MCF-7 cells but coactivators (Co-A) in the Ishikawa cells to the IGF-1 and c-Myc promoters that contain no ERE, strongly suggesting that genes regulated through tethering to AP-1 and Sp-1, not those regulated through direct DNA binding to ERE, mediate the agonistic activity of TAM. In the same report, it was also demonstrated that SRC-1 is differentially expressed at higher level in Ishikawa than MCF-7 cells; that the blockage of SRC-1, but not AIB1, by siRNA inhibited the agonistic activity of TAM in stimulating c-Myc and IGF-1 mRNA expression in Ishikawa cells and that the transfection of SRC-1, not GRIP1 or AIB1, into MCF-7 cells increased the TAM induction of IGF-1 and c-Myc. These suggest that the level of SRC-1 expression may play a critical role in specifying the tissue selective agonistic activity of TAM.

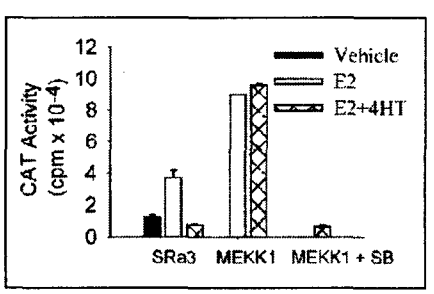


Fig. 1. P38 inhibitor reverses the MEKK1-induced blockage of the antagonistic activity of 4HT in endometrial cancer cells. ER-negative Ishikawa cells were transfected with EREe1bCAT, pLEN-hER and pLENβGal with or without active MEKK1. Transfected cells were treated with vehicle, E2, 4HT or both together with or without SB. CAT activity is assayed and normalized with β-gal.

B. Our studies. Our previous studies (15) have shown that the expression of an active form of MEKK1, a mitogen activated protein kinase kinase kinase (MAPKKK), significantly increased the the transcriptional activity of the ER in endometrial and endometrioid ovarian cancer cells. Mechanistisc studies showed that the MEKK1-induced ER activation is mediated through both JNK and p38/Hog1. More importantly, the expression of the active MEKK1 increased the agonistic activity of 4-hydroxytamoxifen (4HT) to a level comparable to that of E2 and fully blocked its antagobnistic activity. These studies suggest that the uterine specific agonistic activity of TAM may be determined by the status of kinases acting downstream of MEKK1 such as p38/Hog1 and JNK.

This prediction is substantiated by subsequent studies presented in a manuscript in press (16), in which we showed that E2 activated p38 in an ER-dependent manner in E-Ca cells and that the ER is phosphorylated *in vitro* and *in vivo* by a kinase that binds to and acts downstream of p38. The phosphorylation site was identified to be a novel site, threonine-311 (Thr^{311}) in the Helix 1 of the hormone-binding domain. The mutation of Thr^{311} to alanine did not affect estrogen binding of the ER but compromised its interaction with Co-A. Suppression of p38 activity or mutation of the site inhibited the E2-induced receptor nuclear localization as well as its transcriptional activation by E2 and MEKK1. More interestingly, our data in this manuscript showed that 4HT, but not ICI

182,780, also stimulated p38 activity in an ER-dependent manner in Ishikawa cells and that the mutation of Thr^{311} phosphorylation eliminated SRC-1 binding but only modestly reduced TIF2/GRIP1 binding. As mentioned earlier, SRC-1 expression was correlated to TAM activity (). Thus, the preferential regulation of SRC-1 binding by Thr^{311} phosphorylation suggests that the phosphorylation may be another important determinant for the agonistic activity of TAM.

Consistent with this idea, our further studies showed that a p38 inhibitor, SB203580 (SB), recovers the antagonistic activity of 4HT from MEKK1-mediated blockage in transcriptional assays in Ishikawa cells (Fig. 1). More importantly, the p38 inhibitor suppressed the agonistic activity of 4HT and E2 in stimulating endogenous gene expression (Fig. 2, upper panel) and Ishikawa cell growth (Fig. 2, lower panel). Interestingly, the basal ER activity in the absence of ligands were not affected by SB in both assays, showing that the effect is not due to general toxicity of the chemical.

Different from thyroid receptors, the ER does not bind SMRT and NCoR unless it is occupied by antagonists like TAM (17) and the interaction with Co-R is thus considered a good indicator for the antagonistic activity. As shown in Fig. 3, the elimination of Thr^{311} phosphorylation by the p38 inhibitor or the mutation of Thr^{311} to an unphosphorylatable aminao acid generated an ER that constitutively bind Co-R in the absence of TAM (Fig. 3). Besides the preferential effect on SRC-1, the constitutive interaction of the dephosphorylated ER with Co-R is another strong evidence that Thr^{311} phopshorylation is an important factor that determines the agonistic activity of TAM.

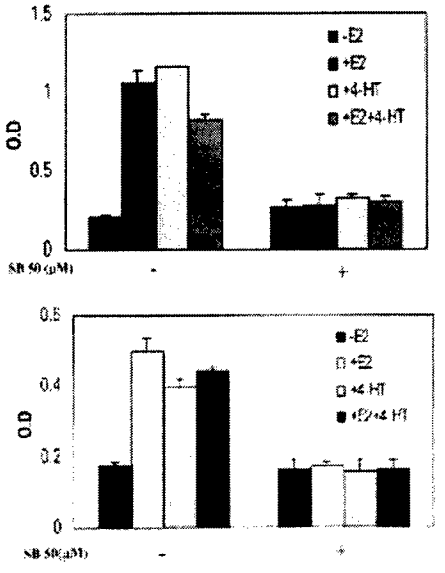


Fig. 2. Suppression of estrogen and tamoxifen induced endometrial cancer cell growth (upper) and endogenous gene expression (lower) by p38 inhibitor. ER-positive Ishikawa cells were treated with Vehicle (-E2), E2, 4HT or E2 + 4HT in the presence or absence of SB. To measure the growth, MTT assays were performed after treating for 3 days and OD595 was read. For alkaline phosphatase assays, cells were treated for 24 hours and OD420 was read after enzyme reactions.

If p38 and Thr^{311} phosphorylation are related to TAM activity, their status may change during the transition of B-Ca from TAM sensitive to resistant status. To determine whether this is the case, we developed a stable TAM-resistant MCF-7 cell line (designated MCF-7R) by stepwise selection of MCF-7 cells in increasing concentration from 10^{-9} to 10^{-6} M in a 3-month period of time and the cells have since been cultured for almost two years in the presence of TAM. Comparing to MCF-7 cells, p38 activity is significantly increased in MCF-7R cells (Fig. 4) and the ER becomes constitutively phosphorylated on Thr residues (Fig. 5). Based on the ATF6 activity (Fig. 4B) which is known to depend on p38 phosphorylation, p38 activity is at least 3 times higher in MCF-7R than MCF-7 cells.

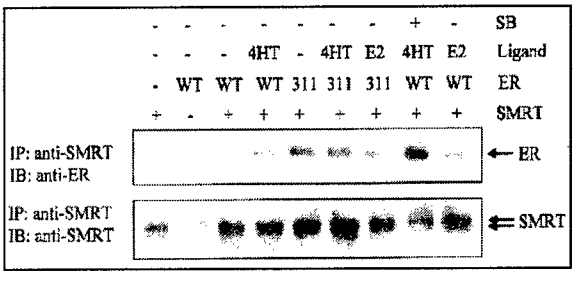


Fig. 3. Constitutive interaction of ER with SMRT in the absence of tamoxifen induced by SB or Thr^{311} mutation. ER-negative Ishikawa cells were transfected with Ala^{311} mutant (311) or wild type (WT) ER with or without SMRT as indicated. IP-Western was performed after the cells were treated for 45 min.

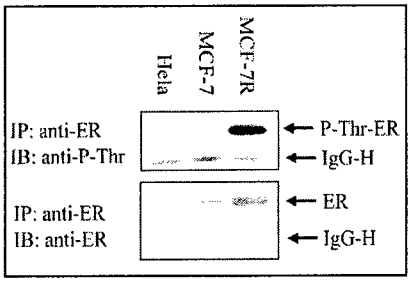


Fig. 5. Increased level of ER Thr phosphorylation in MCF-7R cells. Equal amount of cell lystaes were analyzed by IP-IB. Hela serves as a negative control.

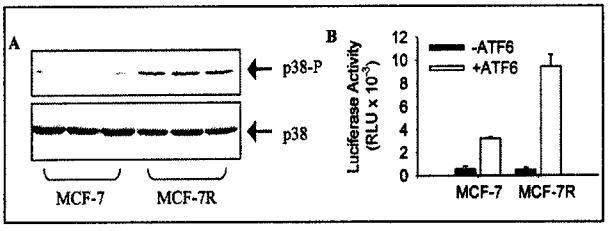


Fig. 4A. Increased phospho-p38 in MCF-7R cells. Triplicate samples of MCF-7 and MCF-7R cells were subjected to IB with either anti-phospho-p38 (top) or anti-p38 (bottom) antibodies. **Fig. 4B. Increased ATF6 activity in MCF-7R cells.** Cells were transfected with Gal4-ATF6, GalLuc and pCMVβGal. Luciferase activity was determined and normalized with β-gal activity. Both MCF-7and MCF-7R cells were cultured in5% charcol stripped FBS for three days without TAM before experiments.

Consistent with the increased p38 activity and Thr^{311} phosphorylation, ER in MCF-7R cells loss the ability to interact with Co-R in the presence of TAM (Fig. 6, upper panel) which is restored by co-treatment with the p38 inhibitor (Fig. 6, lower panel). More importantly, the p38 inhibitor suppressed the agonistic activity of E2 and TAM in stimulating the growth of MCF-7R without an effect on the basal activity in the absence of ligands (Fig. 7), suggesting that it may be a useful drug to treat TAM resistant B-Ca or to prevent the development of resistance.

II. HYPOTHESIS/RATIONALE/PURPOSE

Based on our studies, **we propose that p38 MAPK-mediated ER phosphorylation at Thr^{311} determines the promoter-context specific recruitment of coactivator and corepressors to ER target genes and, through the differential recruitment of transcriptional coregulators, specifies the tissue-specific activity of TAM in endometrial and breast cancer cells. We further propose that increased p38 MAPK activity and Thr^{311} ER phosphorylation directly contribute to breast cancer's resistance to TAM by permitting TAM-bound ER to recruit coactivators to growth-related genes and that the suppression of the p38 MAPK pathway provides a novel therapeurtic target to overcome the TAM resistance**.

The **rationale** is that, in **E-Ca cells**, TAM itself induces ER phosphorylation at Thr^{311} and the TAM-bound, **Thr^{311}-phosphorylated** ER recruits Co-A complex to induce the expression of growth-promoting genes like c-Myc and IGF-1. Therefore, TAM exhibits E2-like agonistic activity. In **B-Ca cells**, due to the lack of TAM induction of ER phosphorylation at Thr^{311}, TAM-bound but **Thr^{311}-dephosphorylated** ER recruits Co-R to inhibit the expression of these growth-promoting genes. Therefore, TAM exhibits antagonistic activity to suppress E2 action. **During the transition to TAM resistant status**, p38 becomes constitutively active in B-Ca cells, which phosphorylates the ER at Thr^{311}. The phosphorylation prevents the TAM-bound ER from recruiting Co-R complex, permitting the recruitment of Co-A complex to enhance the expression of growth promoting genes in a way similar to what happens in E-Ca cells.

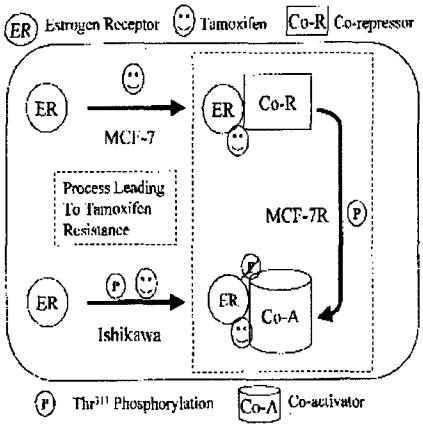
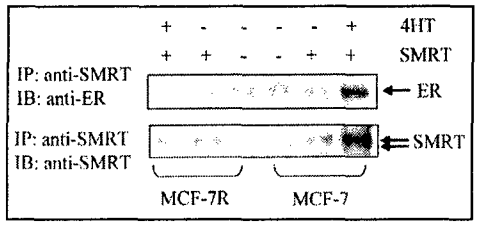


Fig. 8. A hypothetic model explaining the role of ER phosphorylation at Thr^{311} in determining the tissue selective agonistic activity and the development of B-Ca' resistance to tamoxifen.



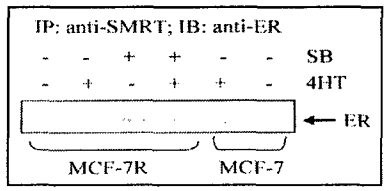


Fig. 6. Loss of tamoxifen-induced ER interaction with corepressors in MCF-7R (upper) and its recovery by treatment with p38 inhibitor (lower). Cells were transfected with or without SMRT and lysates were analyzed by IP-IB as indicated.

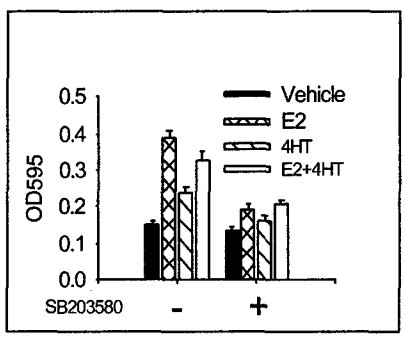


Fig. 7. Suppression of the growth response of MCF-7R to estrogens and tamoxifen by p38 inhibitor. MCF-7R were grown in 5% charcol stripped FBS for 3 days before treatment. Cell growth was determined by MTT assay as described in Fig. 2 for Ishikawa cells.

The **overall purpose** of this grant proposal is to validate this hypothesis and to test, in cell line and animal model systems, whether p38 inhibitors can be used to treat TAM resistant B-Ca or to prevent the development of the resistance.

III. SPECIFIC AIMS

Aim 1. To define the role of Thr^{311} phosphorylation in the promoter-context specific recruitment of transcriptional coregulators and the relationship of the recruitment to the agonistic activity of TAM in inducing the expression of growth related genes as well as the endometrial cell growth.

Aim 2. To define the role of Thr^{311} phosphorylation in the development of B-Ca cells' resistance to TAM by determining whether Thr^{311} phosphorylation is required for the transition of MCF-7 cells from TAM sensitive to resistance and whether the association between the increased Thr^{311}-phoasphorylation and MCF-7R can be translated into TAM resistant tumors induced in animals by systematic administration of TAM.

Aim 3. Using MCF-7 nude mice model and a rat mammary tumor model, to test whether the suppression of p38 MAPK pathway and Thr^{311} phosphorylation offers a new therapeutic target to overcome the resistance of B-Ca to TAM.

IV. METHODS

Aim 1: Our studies so far relies on an indirect approach to detect the ER Thr^{311} phosphorylation. In order to study the role of Thr^{311} phosphorylation in the promoter context-specific coregulator recruitment, it is necessary to develop antibodies that differentially recognize only ER forms phosphorylated or dephosphorylated at Thr^{311}.

1a. To generate Phospho-Thr^{311} specific anti-ER antibodies: In collaboration withe Upstate Biotechnology which is specialized in producing and selling the research community antibodies including many phospho-specific antibodies, we are in the process of generating anti-phospho Thr^{311} ER antibodies using phospho-Thr^{311} synthetic peptide. High titer serum will be affinity purified first against the nonphosphorylated peptides to remove nonspecific antibodies. The passthrough will be purified against phosphorylated peptide. The phospho-Thr^{311} antibodies will be tested for ELISA, immunoblotting (IB), immuoprecipitation (IP) and imunohistochemistry. With the expertise of Upsrtate in antibody production, we expect no problems to generate the antibodies.

1b. To determine whether the phosph-ER interacts only with Co-A and whether the dephospho-ER interacts only with Co-R in TAM-treated Ishikawa cells. Afer the anti-phospho ER antibodies are generated, ER-positive Ishikawa cells will be treated with TAM for 45 minutes and IP-IB experiments as shown in Figures 3 and 6 will be performed using the phospho-specific and total ER antibodies for the IB step. Working with Dr. Ed Haller who is specialized in electronic and confocal microscopic analysis in the Imaging Core in our department, we have demonstrated the different subcellular localization of wild type and Ala^{311} mutant ER (16). We are currently working on the conditions for the immunofluorescence colocalization of ER and Co-A and Co-R using available commercial antibodies and the preliminary confocal images look very promising. After the phospho-specific ER antibodies are generated, we will use the same procedure to show in Ishikawa cells that phospho-ER interacts with only Co-A, not Co-R.

1c. To determine whether the Thr^{311}-phospho-ER is recruited with Co-A to c-Myc and IGF-1 promoters while Thr^{311} dephospho- ER is recruited with Co-R to cathepsin D and EBAG9 in TAM-treated Ishikawa cells by CHIP assays. CHIP assays for ER target genes including c-Myc, IGF-1, cathepsin D and EBAG9 using Co-A and Co-R antibodies have been described (14). ER-positive Ishikawa cells will be treated with TAM for 45 minutes and CHIP assays will be performed using the Thr^{311} phospho-ER antibodies generated in **Aim 1a** for the IP step. We expect that phosphorylated form of ER, not dephosphorylated, is recruited to c-Myc and IGF-1 promoters together with SRC-1 and other Co-A and that de-phosphorylated ER, not phosphorylated ER, will be recruited to cathepsin D and EBAG9 promoters. Ishikawa cells treated with E2 will be included as controls and phosphorylated ER will be expected to recruited to all four genes with Co-A.

1d. To determine whetherthe inhibition of p38 MAPK eliminates the recruitment of phospho-ER and Co-A and promotes the recruitment of dephospho-ER and Co-R to the promoters of c-Myc and IGF-1 and as well as TAM induction of c-Myc and IGF-1 expression. The same CHIP assays described in **Aim 1c** will be performed in the presence of SB203580. It is expected that in the presence of the p38 inhibitor, dephosphorylated ER and Co-R will be recruited to c-Myc and IGF-1 promoters. The induction of c-Myc and IGF-1 mRNA by TAM will be determined by Northern blotting (14) in Ishikawa cells after being treated with TAM for 24 hours in the presence or absence of the p38 inhibitor. It is expected that the IGF-1 and c-Myc induction will be suppressed by the p38 inhibitor.

Alternate strategy: If the antibodies, for any technical reasons, are not generated on time, Ala^{311} and Glu^{311} mutant ER will be stably tranfected into ER-negative Ishikawa cells. The two cell lines will be treated with TAM and E2 and similar CHIP assays will be performed. Ala^{311} ER is expected to behave like dephosphorylated ER and to be recruited together with Co-R to c-Myc and IGF-1 promoters after TAM and E2 treatment. Glu ER should mimic the phospho-ER and is expected to be recruited with Co-A to c-Myc and IGF-1 promoters. If Glu mutation fails to generate active form of the ER as it has been shown for some proteins, active MKK6b, a p38 specific MKK we obtained from our collaborator Dr. Bing Su at MD Anderson Cancer Center, will be transfected into ER-positive Ishikawa cells. In the resulting cell, the ER will be constitutively phosphorylated at Thr^{311}. The same set information is expected to be obtained in the CHIP assays and colocalization studies with the stable cell lines. Ala^{311} mutant ER has been used in our studies (16) and Glu^{311} ER mutant is currently being generated.

Aim 2: Similar to the ER-negative and ER-positive Ishihara cells used in our studies (15, 16), Dr. Adrian Lee's group at Baylor College of Medicine reported the establishment of an ER-negative MCF-7 cell line in which the reexpression of ER restores the sensitivity of the cells to E2 and IGF-1 (18). Dr. Lee agrees to provide the cells for our studies with Thr^{311} mutant receptors.

2a. To establish stable transfectant of ER-negative MCF-7 cells with Ala^{311} and Glu^{311} mutant ER and to compare the cells' growth response to E2 and IGF-1 as well as their ability to develop TAM resistance during stepwise selection with increasing concentrations of TAM. The ER-negative MCF-7 cells will be stably transfected with Ala^{311} or Glu^{311} mutant ER and their ER expression and response to E2 and IGF-1 will be compared with the wild type ER transfected cells and the parental MCF-7 cells. Then, the established cells will be used to test whether the stable expression of Ala^{311} mutant ER will create a MCF-7 cell that will not allow TAM resistance to develop or take long time to develop the phenotype and whether the Glu^{311} mutant will create a cell that are TAM resistance to begin with or easy to become TAM resistant.

2b. To determine whether constitutive p38 activation by the stable expression of an active MKK6 in MCF-7 cells results in TAM resistance and whether the p38 inhibitor prevents the transition of MCF-7 cells to TAM resistant status. To test whether the constitutive p38 activity is sufficient to induce TAM resistant phenotype, MCF-7 cells will be stably transfected with active MKK6. MKK6b expression and p38 activity will be tested by IB with M2 (MKK6b is Flag-tagged) and anti-phospho p38 antibodies, respectively. The stable transfectants containing high level of p38 activity will be chosen to test for TAM resistancy. To test whether p38 is required for the development of TAM resistance, MCF-7 cells will be selected against increasing concentration of TAM in the presence or absence of SB203580. In the presence of SB compound, it is expected that TAM resistant cells will not arise or the development will be delayed.

2c. To determine whetherthe increasedp38 and Thr^{311} phosphorylation associated with the development of MCF-7R is true for TAM resistant B-Ca induced in whole animals by systematic administration of TAM .

2c(1). MCF-7 in nude mice: Following the procedure described by Osborne et al (19), MCF-7 cells will be injected into nude mice to allow the tumor formation in the presence of E2 pellets (0.25 mg/pellet) which releases 17β-estradiol at steady rate. After 4 weeks, TAM sensitive tumors are expected to form in 90% of mice and the average diameter of the tumor will be around 7 mm. The sensitive tumors will be separated into two groups. In one group the tumor will be allowed to grow to 10 mm size and mice will be sacrificed for tumor collection. In the other group, E2 pellets will be replaced with TAM pellets (5 mg/mouse) and TAM resistant tumors are expected to regrow after a 3-4 month period in which the tumors are stable. After the TAM resistant tumors reach the same size as the sensitive ones, the mice will be sacrificed and the tumors will be collected. The two group tumors will be subjected to ELISA analysis using the commercial kit for phospho-p38 and total p38 (BioSource International, Inc) since they are highly sensitive and quantitative. To analyze the level of Thr^{311} ER phosphorylation, the samples will be subjected to IB with the phospho- Thr^{311} antibody generated in **Aim 1a**. Total ER level will be determined by IB using F-10 antibody used in our studies (Fig. 5).

2c(2). Sample size and statistics. Working in collaboration with David Cuthbertson, M.S., a biostatistician in Moffitt Cancer Center Biostatistics Core, we predict that 15 pairs of TAM sensitive and resistant tumors are needed for the tumor studies. This is based on the data in Figure 4 which showed that p38 activity in TAM resistant cells is about 3 times of that in sensitive samples. Given an alpha of 0.05, a standard deviation of 15% of the signal in TAM resistant samples, a sample size of 15 will yield an 95% statistical power. After the experiment is completed, *t*-test will be used to determine if the two groups differ significantly at their level of phospho-p38 and Thr^{311} phospho-ER. All statistical analyses performed will be two-sided using a significance level of 0.05, and conducted by the Moffitt Cancer Center's Biostatistical Core using SAS.

Alternative strategy: Besides the increase in p38 activity demonstrated in Figure 4, JNK (20, 21) and ERK (22) activity was also reported to be increased in TAM resistant cells, which we confirmed in our MCF-7R cells (data not shown). Therefore, increased p38 alone may or may not drive MCF-7 cells to TAM resistant status. If p38 is not sufficient, we will test whether the simultaneous increase of p38 with JNK or ERK or all three MAPKs together will confer TAM resistance. Active JNKK or MEK will be transfected together with MKK6b into MCF-7 cells to test this possibility.

We are in the process of collecting ER-positive primary breast carcinomas from patients who have not gone through TAM treatment and TAM resistant tumors from patients whose resistance developed after an initial objective (partial or complete) response of their primary ER-positive cancers to TAM. Samples from both groups will be analyzed as described for MCF-7 tumors in nude mice. Similarly, TAM-resistant and sensitive rat tumors from **Aim 3b** will also be analyzed if our anti-phospho-ER antibodies and the p38 ELISA kit can be used for rat systems. If the ELISA test has problems for the analysis of p38 activity we will compare the level using Western blot with anti-phospho p38 and anti-total p38 as in Figure 4A. In vitro immunocomplex kinase assay using gamma-^{32}P-ATP will be performed to determine the p38 activity if it becomes necessary. If our anti-phospho ER antibody has problems, the level of Thr^{311} phosphoryaltion of ER will be determined by IP-IB procedure as described in Figures 4 and 5. Fluorescence-based ECL detection technique will be used since it is more quantitative.

Aim 3. Our data suggest that p38 inhibitor suppresses ER activity in reporter assays and as wel as E2 and TAM-induced E-Ca and B-Ca cell growth (Figs. 1, 2 and 7), suggesting that p38 inhibitor may be used for the therapeutic

treatment of both TAM-sensitive and resistant B-Ca. The lack of an effect on the basal activity is understandable since the p38 is a stress-activated protein kinase and may have non-essential function under non-stress conditions. SB203580 has been used for *in vivo* experiments in both rat and mouse disease models including inflamatation, arthritis, septic shock, and myocardial injury (23). No apparent side-effect or toxicity is associated with long-term treatment for weeks at dosages that was shown to inhibit *in vivo* p38 activity (24). The dosage can be used as high as 100 mg/kg/day (23). Based on the inhibition of TNF-α production induced by LPS, IC50 is around 25 mg/kg (23).

There are two animal models frequently used for the investigation of issues related to E2/TAM sensitivity of mammary tumors. One is the MCF-7 nude mice model mentioned earlier and the other one is the carcinogen-induced mammary rat tumors. The MCF-7 tumor in nude mice represents tumors derived from human cells but is from a single cell line. Rat tumors is a real *in vivo* model but it is not human tumor. Therefore, both will be tested for studies in Aim 3.

3a. To test the effect of the p38 inhibitor on MCF-7 tumors generated in nude mice. TAM sensitive and resistant MCF-7 tumors generated in nude mice as described in **Aim 2c(1)** will be treated with SB203580 alone or together with TAM. SB203580 at 10, 30, and 60 mg/kg/day will be administered by *p.o.* gavage into mice daily for three weeks. Tumor growth will be monitored and compared with those with TAM pellet alone. Results with TAM sensitive tumors should reveal whether p38 inhibitor prevent or slow the development of TAM resistance while the result from the TAM resistant group will tell whether p38 inhibitor can be used to treat breast tumors after they become hormone independent.

3b. To test the effect of p38 inhibitor on rat mammary tumors induced by DMBA. DMBA (5 mg/rat/week) will be dissolved in seasame oil and administered to 7 to 8 week old Sprague-Dawley rats once a week for 4 weeks. This procedure described by Fendl and Zimniski (25) is modified from the classical Huggins model (26) and was shown to induce more palpable mammary tumors as early as three weeks after the last administration of the carcinogen. 5 weeks after the first DMBA injection, TAM (1 mg/kg body weight/day, twice daily s.c. injection in 0.1 ml seasame oil) will be administered s.c. daily for 3 weeks. About 25% of the tumors are expected to re-grow and are TAM resistant. To test the prevention of TAM resistance, SB203580 at 10, 30, and 60 mg/kg/day will be administered by *p.o.* gavage alone or together with TAM twice daily starting 5 weeks after the first DMBA injection. To test the effect of SB on TAM resistant tumors, SB alone or together with TAM will be administered after TAM-resistant tumors regrow. The SB treatment for the resistant tumors will be for three weeks also.

Similar to the MCF-7 nude mice experiments, the response of the TAM sensitive tumors in rats will reveal whether the cotreatment with p38 inhibitor prevent or slow the development of TAM resistant tumors while the information from those TAM resistant ones will tell whether p38 inhibitor can overcome the resistance.

3c. Sample size, Data analysis and Statistics. Since no preliminary studies with p38 inhibitor have been done in mammary tumor model, it is hard to predict the exact number of mice and rats for each treatment. We propose to use 10 for each group based on similar studies reported in the literature. The number may have to be adjusted for the repeating experiments based on the statistical analyses of the first test. The size of the tumors will be measured twice weekly after the co-treatment with SB compounds and TAM starts. Tumor volume will be calculated according to $V=(4/3)\pi R1^2R2$ (radius R1<R2). Average tumor volume and percentage change in volume /day will be calculated in each group. All experiments will be performed at least twice and data will be reported as mean ± SEM. Difference between groups will be evaluated with student *t* test. All analyses performed will be two-sided using a significance level of 0.05, and conducted by the Moffett Cancer Center's Biostatistical Core using SAS.

Alternative strategy: As mentioned earlier, besides p38, ERK and JNK activity are also increased in MCF-7R cells (data not shown). If p38 inhibitor alone did not have much effect on TAM resistance, future studies will explore the effect of the combinational inhibition of all three MAPKs together on tamoxifen resistant breast cancers. Like SB compound, JNK and ERK inhibitors are both commercially available.

V. INNOVATION AND SIGNIFICANCE.

Our hypothesis and the objectives under testing are examining the mechanism underlying the development of TAM resistancy and the side effect of TAM in stimulating endometrial cancer formation, which are two of the most important clinical problems associated with breast cancer therapy with tamoxifen. Our theory predicts that the blockage of the p38 MAPK pathway may prevent the development of tamoxifen resistance and eliminating the side effect in endometrial cancer formation, thus enhancing the efficacy of tamoxifen treatment. If our hypotheis is proven by studies proposed in the current application and the main objectives are achieved, our investigation may lead to the development of novel therapeutic strategy to overcome the tamoxifen resistance which is a major health care problem for the clincal management of human breast cancer.

ABBREVIATIONS

B-Ca:	Breast Cancer
E2:	Estrogen or 17β-estradiol
ER:	Estrogen Receptor
SERM:	Selective Estrogen Receptor Modulator
ERα:	Estrogen receptor alpha
ERβ:	Estrogen Receptor beta
TAM:	Tamoxifen
E-Ca:	Endometrial Cancer
CHIP:	Chromatin Immunoprecipitation
Co-R:	Corepressor
ERE:	Estrogehn Response Element
Co-A:	Coactivator
IGF-1:	Insulin-like Growth Factor 1
c-Myc:	Cellular Myc Oncogene
AP-1:	Activating Protein-1
Sp-1:	GC Box-binding Transcription Factor
SRC-1:	Steroid Receptor Coactivator 1
GRIP1:	Glucocorticoid receptor Interacting Protein 1
AIB1:	Amplified In Breast Cancer 1
MEKK1:	MEK Kinase 1
MAPKKK:	Mitogen-activated Protein Kinase Kinase Kinase
JNK	Jun N-terminal Kinase
p38/Hog1:	38 kD MAPK Hopmologous to Yeast Osmosensing Protein Hog1
4-HT:	4-hydroxytamoxifen
TIF2:	Transcriptional Intermediary Factor 2
SB:	SB 203580, a p38 inhibitor
SMRT:	Silencing Mediator of Retinoid and Thyroid Hormone Receptors
NCoR:	Nuclear Receptor Corepressor
MCF-7R:	MCF-7 Cells Resistant to Tamoxifen
ATF6:	Activating Transcription Factor 6
IB:	Immunoblotting
IP:	Immunoprecipitation
IP-IB:	Immunoblotting Following Immunoprecipitation
ELISA:	Enzyme Linked Immunosorbent Assay
EBAG9:	Encoding ER Binding Fragment-associated Antigen 9

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MEKK1-JNK/P38 PATHWAYS AND THE RESISTANCE OF BREAST CANCER CELLS TO TAMOXIFEN

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ABSTRACT: Begin single-space abstract here. Abstract MUST NOT EXCEED ONE PAGE. Abstracts will appear in the proceedings EXACTLY as submitted.

Tamoxifen (TAM) treatment is the preferred first line therapy for estrogen receptor (ER)-positive breast cancers, but patients acquire resistance during the treatment and the mechanism underlying the resistance is not clear. It is known that many TAM-resistant breast cancers still express the ER and MCF-7 tumors which become resistant to TAM use TAM as a mitogenic stimulus, suggesting that the TAM-resistant breast cancer cells may have gained some of the characteristics of uterine cells in which TAM is naturally an ER agonist. Published studies in our lab found that, in endometrial and endometrioid ovarian cancer cells, the expression of an active MEKK1 decreased the antagonistic activity of TAM and increased its agonistic activity. Therefore, we hypothesized that the aquired resistance of breast cancer cells to TAM during the treatment may be due to the increased activity or expression level of MEKK1 or its downstream kinases. Here, we report ERalpha-dependent p38 activation by estrogens and TAM in endometrial adenocarcinoma cells. The activated p38 was found to phosphorylate the ER in vitro and in vivo at a novel residue. Suppression of p38 activity or mutation of the site inhibited the Crm1-dependent nuclear export of the receptor as well as its transcriptional and biological activities. More importantly, TAM-resistant MCF-7 breast cancer cells were found to contain increased p38 activity and to have gained the ability to respond to MEKK1 for inhibition of the antagonistic activity of TAM. Our studies establish the signaling through MEKK1-p38 pathways as a critical determinant for the agonistic activity of estrogens and TAM in breast and endometrial cancer cells. The data supports our original hypothesis that signaling through MEKK1-JNK/p38 pathways contributes to the resistance of breast cancer cells to TAM.

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Regulation of Estrogen Receptor Nuclear Export by Ligand-Induced and p38-Mediated Receptor Phosphorylation

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Estrogen receptors are phosphoproteins which can be activated by ligands, kinase activators, or phosphatase inhibitors. Our previous study showed that p38 mitogen-activated protein kinase was involved in estrogen receptor activation by estrogens and MEKK1. Here, we report estrogen receptor-dependent p38 activation by estrogens in endometrial adenocarcinoma cells and in vitro and in vivo phosphorylation of the estrogen receptor α mediated through p38. The phosphorylation site was identified as threonine-311 (Thr^{311}), located in helix 1 of the hormone-binding domain. The mutation of threonine-311 to alanine did not affect estrogen binding of the receptor but compromised its interaction with coactivators. Suppression of p38 activity or mutation of the site inhibited the estrogen-induced receptor nuclear localization as well as its transcriptional activation by estrogens and MEKK1. The inhibition of the p38 signal pathway by a specific chemical inhibitor blocked the biological activities of estrogens in regulating endogenous gene expression as well as endometrial cancer cell growth. Our studies demonstrate the role of estrogen receptor phosphorylation induced by the natural ligand in estrogen receptor's cellular distribution and its significant contribution to the growth-stimulating activity of estrogens in endometrial cancer cells.

Estrogens are female sex steroid hormones that control development, maintenance, and regulation of the female reproductive phenotype and behavior. They also stimulate the growth of normal and transformed epithelial cells of the female reproductive systems. The effect of estrogens is mediated through both estrogen receptors α and β (ERα and ERβ), which belong to the nuclear hormone receptor superfamily, a group of ligand-regulated, zinc finger-containing transcription factors (11, 40). The superfamily includes not only receptors for classical steroids such as estrogens, androgens, progesterones, and glucocorticoids, but also receptors for steroid analogues and nonsteroid ligands such as vitamin D, thyroid, and retinoic acids, as well as orphan receptors for which the ligand is unknown.

Unlike the thyroid and vitamin D receptors, which reside in the nucleus in the absence of ligands, receptors for classical steroids such as ERα are targeted to the nucleus after binding with estrogens or selective estrogen receptor modulators such as tamoxifen. In contrast, the pure ERα antagonist ICI 182,780 directs the ERα to the cytoplasm (6). Of importance in this respect are three clusters of basic amino acids, similar to the nuclear localization signals found in simian virus 40 large T antigen, which were identified in the DNA binding domain and the hinge region of ERα (44). The nuclear localization signals are constitutively active and do not seem to explain the estrogen effect on the ERα nuclear localization (44). It is therefore possible that estrogen-induced targeting of ERα to the nucleus is mediated through other mechanisms.

Studies in recent years have provided increasing evidence that nuclear localization is also controlled through nuclear export signals (17, 38, 42). A number of studies on exported proteins have shown that typical nuclear export signals are hydrophobic, leucine-rich sequences that signal the nuclear export complex containing exportin/Crm1 and RanGTP to transfer nuclear export signal-carrying proteins to the cytoplasm (8, 12, 29, 37).

Besides ligands, nonsteroid stimuli such as kinase activators, phosphatase inhibitors, neurotransmitters, and growth factors were also shown to activate the ERα (35). They either activate the receptor ligand independently or enhance the ligand-induced activity. Consistent with the cross talk with kinase/phosphatase pathways, ERα has been found to be phosphorylated at different sites by various kinases, including the external signal-regulated kinase (4, 19), cyclin A-CDK2 (32), c-SRC (25), protein kinase A (5), and $pp90^{RSK1}$ (16). Except for tyrosine-537, all known ERα phosphorylation sites are on serine residues (1). Single-site mutation or simultaneous mutation at multiple sites reduced the transcriptional activity of the receptor (13). With the exception of the serine-236 phosphorylation by protein kinase A (5), most studies indicated the general role of ERα phosphorylation to be the regulation of the transcriptional activity of the receptor by modulating the interaction between the ERα activation domains and transcriptional coactivators (9, 28, 39).

In the present studies, we report that, in ERα-expressing endometrial cancer cells, 17β-estradiol activates the p38 mitogen-activated protein kinase (MAPK) pathway, which in turn mediates the phosphorylation of the ERα on threonine-311 (Thr^{311}), promoting the receptor's nuclear localization and interaction with steroid receptor coactivators. Additional studies show that Thr^{311} phosphorylation in ERα is a critical determinant for its transcriptional and biological activities in endometrial cancer cells.

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MATERIALS AND METHODS

Plasmids. pLEN-hERα (2, 36), pLENβgal (36), Flag-p38 (43), SRαMEKK1 (CT) (26), and EREe1bLuc have been described (20, 36). pBind expression vectors for SRC1 and TIF2 have been described (24). T311A was generated by site-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene, La Jolla, Calif.) and confirmed by direct sequencing. pLEN-hERα was used as the template for the mutagenesis. The primers for the PCR were synthetic oligonucleotides with the sequences 5′-CCTTGTCCCTGGCAGCCGACCAGATG-3′ and 5′-CATCTGGTCGGCTGCCAGGGACAAGG-3′.

Transfection and reporter assays. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection was performed with Lipofectamine (Gibco-BRL Life Technologies, Rockville, Md.) as described previously (20). After transfection, cells were placed in Dulbecco's modified Eagle's medium containing 5% charcoal-stripped fetal bovine serum, treated with different reagents, and assayed for luciferase and β-galactosidase activity as described previously (21). ERα transcriptional activity was measured by normalizing the luciferase activity to the corresponding β-galactosidase activity.

Immunoprecipitation and immunoblotting analysis. Detection of Flag-p38 and ERα expression by immunoblotting has been described (20). To determine the level of Thr phosphorylation of ERα, cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 5 mM sodium fluoride, and protease inhibitor cocktail. The lysates were immunoprecipitated with F-10 anti-ERα monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.). The ERα precipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8% polyacrylamide), transferred to nitrocellulose, probed with the antiphosphothreonine antibody (Sigma, St. Louis, Mo.) overnight, and visualized with the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.).

To detect the binding of coactivators to ERα, Ishikawa cells were transfected with pBind-SRC1, pBind-TIF2, or pBind as a control vector together with either wild-type or mutant ERα. Following immunoprecipitation with anti-ERα F-10 antibody, coactivator in the precipitates was detected with anti-Gal4 DNA-binding domain antibody (Santa Cruz).

In vitro kinase assays. p38 in vitro immunocomplex kinase assays were described before (20). For in vitro kinase assays with purified p38 kinase, recombinant ERα protein was incubated with 25 ng of active recombinant human p38α/SAPK2a (2.4 U/μg; Upstate Biotechnology, Lake Placid, N.Y.).

Phosphoamino acid analysis, phosphopeptide mapping, and Edman degradation. Phosphoamino acid analysis, phosphopeptide mapping, and manual Edman degradation were performed as described previously (5, 13, 33).

Hormone binding assays. Cells were incubated for 2 h with ^{3}H-labeled 17β-estradiol (New England Nuclear, Boston, Mass.) at the indicated concentrations. To determine nonspecific binding, a 200-fold excess of unlabeled diethylstilbestrol (DES; Sigma, St. Louis, Mo.) was added in addition to ^{3}H-labeled 17β-estradiol in a parallel set of samples. Cells were washed five times with ice-cold PBS and extracted in ethanol, and radioactivity was counted in a scintillation counter. Specific binding was calculated by subtracting nonspecific binding in cells incubated with excess DES from the total binding in cells incubated with [^{3}H]17β-estradiol alone. Each data point was analyzed in duplicate.

Preparation of nuclear and cytoplasmic extracts. For preparation of nuclear and cytosolic extracts, cells were scraped into hypotonic buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_2P_2O_7$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, and protease cocktail. Nuclei were separated from the cytosol by centrifugation at 11,750 × *g* for 20 min at 4°C. After centrifugation, pellets containing nuclei were resuspended in the same hypotonic buffer but containing 420 mM KCl and 20% glucose. To be representative of the ERα distribution in a cell, the same volume of buffer was used to prepare the nuclear and cytoplasmic fractions for each preparation, and equal volumes of cytosolic and nuclear extracts were subjected to immunoblotting analyses.

Immunofluorescence staining. Ishikawa cells were seeded onto chamber slides and transfected with 0.1 μg of either wild-type or T311A mutant ERα expression constructs. Forty-eight hours later, the cells were fixed in 2% paraformaldehyde, permeabilized with phosphate-buffered saline (PBS) containing 1% Triton X-100 and 1% bovine serum albumin, and blocked in blocking buffer (PBS containing 1% bovine serum albumin and 0.1% NP-40). Cells were then incubated with anti-ERα antibody F-10. After extensive washing with PBS, anti-mouse immunoglobulin-fluorescein isothiocyanate (FITC) conjugate (Sigma, St. Louis, Mo.) was applied in blocking buffer as the secondary antibody. Nuclei were stained with 2′,6′-diamidino-2-phenylindole (DAPI) in antifade mounting medium (Vector laboratories, Burlingame, Calif.) before analysis under a fluorescence microscope.

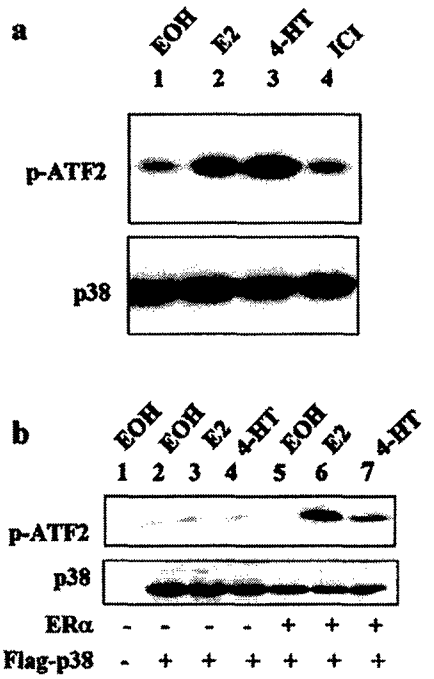

FIG. 1. 17β-Estradiol and 4-hydroxytamoxifen activate p38 MAPK in endometrial cancer cells. (a) Activation of p38 by ERα agonists but not pure antagonist. ERα-negative Ishikawa cells were transfected with 0.5 μg of Flag-p38 and 0.5 μg of pLEN-hERα and treated with 10^{-8} M 17β-estradiol (E2), 10^{-6} M 4-hydroxytamoxifen (4-HT), 10^{-7} M ICI 182,780 (ICI), or ethanol (EOH) as a vehicle control for 30 min. The kinase activity was determined by immunocomplex kinase assays with GST-ATF2 as a substrate (upper panel). The level of Flag-p38 expression was determined by Western blotting with the M2 antibody (lower panel). p-ATF2, phosphorylated GST-ATF2. (b) ERα dependency of p38 activation by ERα agonists. ERα-negative Ishikawa cells were transfected with Flag-p38 and pLEN-hERα or control vectors. Cells were treated and the p38 activity was assayed as explained for panel a.

Alkaline phosphatase and MTT assays. Alkaline phosphatase activity was determined in ERα-positive Ishikawa cells as described previously (22). Cell growth was measured in methylthiazole tetrazolium (MTT) assays as described previously (21).

RESULTS

Activation of p38 by estrogens in endometrial adenocarcinoma cells. Our previous studies (20) showed that estrogens require p38 to activate ERα in endometrial cancer cells. To determine whether estrogens activate p38 in these cells, Ishikawa cells lacking ERα expression (20) were transfected with ERα and Flag-p38 expression vectors. Following treatment with 17β-estradiol, 4-hydroxytamoxifen, or ICI 182,780, the p38 protein was immunoprecipitated with the M2 anti-Flag antibody, and its activity was determined by immunocomplex kinase assays with glutathione *S*-transferase (GST)-ATF2 as a substrate. As shown in Fig. 1a, treatment with 17β-estradiol induced p38 activity (Fig. 1a, lane 2 of top panel) compared to the vehicle control (Fig. 1a, lane 1 of top panel). Interestingly, 4-hydroxytamoxifen also increased the p38 activity (Fig. 1a, lane 3 of top panel), supporting our previous data showing the

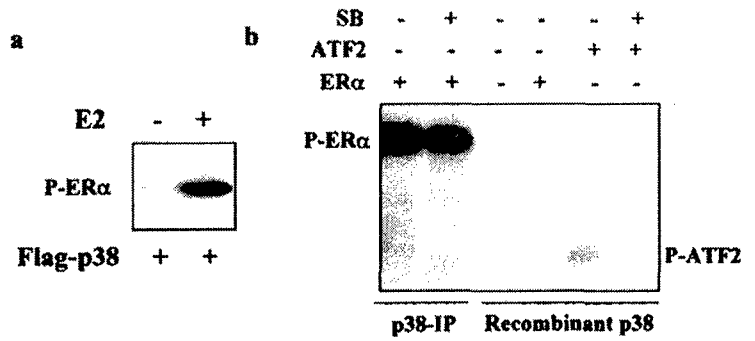

FIG. 2. Phosphorylation of purified ERα protein by p38 immunocomplex isolated from estrogen-treated cells. (a) ERα-negative Ishikawa cells were transfected with 0.5 μg of Flag-p38 and 0.5 μg of pLEN-hERα and treated with 17β-estradiol (E2) or not for 30 min. The phosphorylation of recombinant ERα protein by p38 immunoprecipitates was determined in in vitro immunocomplex kinase assays. P-ERα, phosphorylated ERα. (b) Lack of ERα phosphorylation by purified recombinant p38. Phosphorylation of recombinant ERα by p38 immunocomplex (p38-IP) or recombinant human p38α purified from bacteria in the presence or absence of 50 μM SB 203580 (SB) was determined. As controls, recombinant ATF2 was phosphorylated by purified p38, and the phosphorylation was inhibited by SB 203580.

involvement of the MEKK1-p38 MAPK pathway in the uterine-specific agonistic activity of tamoxifen (20). On the other hand, the pure ERα antagonist ICI 182,780 did not induce p38 activity (Fig. 1a, lane 4 of top panel). The level of p38 expression was not affected by the hormonal treatment (Fig. 1a, bottom panel), demonstrating that 17β-estradiol and 4-hydroxytamoxifen increased its specific activity.

To show that p38 activation by estrogens requires ERα, the induction of p38 activity by 17β-estradiol or 4-hydroxytamoxifen was assayed in the ERα-negative Ishikawa cells transfected or not with ERα. As shown in Fig. 1b, 17β-estradiol and 4-hydroxytamoxifen did not affect p38 activity in the absence of ERα (Fig. 1b, lanes 3 and 4), whereas in ERα-transfected cells, treatment with either 17β-estradiol (Fig. 1b, lane 6) or 4-hydroxytamoxifen (Fig. 1b, lane 7) again increased p38 activity compared to the vehicle control (Fig. 1b, lane 5). No phosphorylation of ATF2 was detected in the absence of Flag-p38 (Fig. 1b, lane 1), showing the specificity of ATF2 phosphorylation to p38. These data demonstrate that ERα agonists activate p38 via ERα in endometrial cancer cells.

Phosphorylation of ERα by p38 immunocomplex but not recombinant p38 purified from bacteria. Since MEKK1-activated p38 immunoprecipitated from cells phosphorylated the recombinant ERα protein in in vitro immunocomplex kinase assays (20), we next tested whether p38 immunoprecipitates from estrogen-treated cells would also phosphorylate the ERα protein. ERα-negative Ishikawa cells were transfected with p38 and ERα expression vectors, and p38 immunocomplex kinase assays were performed as described for Fig. 1 with the exception that recombinant human ERα protein was used as a substrate. As shown in Fig. 2a, the ERα protein was weakly phosphorylated by the basal p38 immunoprecipitated from cells treated with vehicle (Fig. 2a, lane 1). Treatment with 17β-estradiol induced ERα phosphorylation considerably (Fig. 2a, lane 2), suggesting that the ERα protein is a substrate for the p38 immunocomplex isolated from estrogen-treated cells.

To determine whether it is the p38 or an associated kinase that phosphorylates ERα, the phosphorylation of ERα protein was tested with active p38α purified from bacteria. As shown in Fig. 2b, while recombinant p38 phosphorylated ATF2, which was blocked by SB 203580, it did not phosphorylate ERα in parallel reactions. In parallel analysis, p38 immunocomplexes phosphorylated ERα protein, as expected, but p38 inhibitor added to the kinase reactions did not block this phosphorylation. These analyses suggest that it is a p38-associated kinase that phosphorylated ERα in the immunocomplex.

p38-mediated phosphorylation of ERα on Thr residues in vitro and in vivo. In previous studies (20), none of the presently known ERα phosphorylation sites were found to be required for ERα activation by MEKK1, suggesting that p38 MAPK may induce the phosphorylation of the ERα at novel sites. To determine which type of amino acid is phosphorylated, ERα protein phosphorylated in vitro by p38 immunocomplex (Fig. 2a, lane 2) was excised from the SDS-PAGE gel and subjected to phosphoamino acid analysis. The result re-

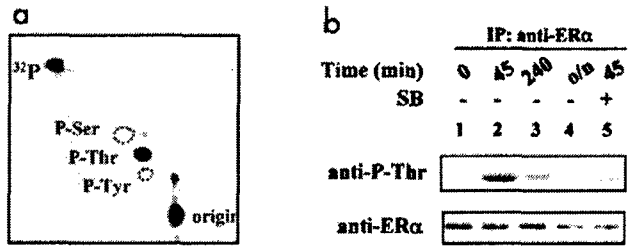

FIG. 3. ERα is phosphorylated by the p38 immunocomplex on Thr residues. (a) Phosphoamino acid analysis. ERα phosphorylated by the p38 immunocomplex was treated with HCl for hydrolysis, mixed with phosphoamino acid markers, and separated by two-dimensional electrophoresis on a TLC plate. The positions of phosphoamino acids from the markers (indicated by dashed circles) were revealed by ninhydrin spray, and ^{32}P-radiolabeled phosphoamino acids from the sample were located by autoradiography. ^{32}P, free ^{32}P from the hydrolysis; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. (b) p38-mediated ERα phosphorylation on Thr residues in vivo induced by 17β-estradiol. ERα-positive Ishikawa cells were treated with 10^{-8} M 17β-estradiol for the indicated times with 50 μM SB 203580 (SB) or vehicle. o/n, overnight. The Thr phosphorylation of endogenous ERα protein was detected by Western blotting with antiphosphothreonine antibody (anti-P-Thr) (upper panel) following immunoprecipitation with the F-10 anti-ERα antibody. The level of ERα in the immunoprecipitates was determined by immunoblotting with the same F-10 antibody (lower panel).

vealed that ERα phosphorylation by p38 occurs exclusively on Thr residues (Fig. 3a).

Since the p38 immunocomplex phosphorylated ERα in vitro exclusively on Thr residues, in contrast to all known ERα phosphorylation sites, we examined whether estrogens induce Thr phosphorylation of ERα in vivo with an antibody that specifically recognizes proteins containing phosphorylated Thr residues. ERα-positive Ishikawa cells (30) were treated with 17β-estradiol for various time periods. The endogenous ERα protein was then immunoprecipitated with an anti-ERα antibody and subjected to immunoblotting with either the anti-ERα or the antiphosphothreonine antibody. As shown in Fig. 3b, treatment with 17β-estradiol for 45 min resulted in a considerable increase in Thr phosphorylation of the endogenous ERα protein (Fig. 3b, lane 2) compared to the control (Fig. 3b, lane 1). The Thr phosphorylation returned to the basal level after the cells were treated for 4 h (Fig. 3b, lane 3) or overnight (Fig. 3b, lane 4), indicating that it is a transient event. Treatment of cells with SB 203580 blocked in vivo ERα phosphorylation on Thr residues (Fig. 3b, lane 5), demonstrating that the endogenous p38 MAPK pathway in endometrial cancer cells is required for the phosphorylation. Compared with the decreased level of the ERα protein (Fig. 3b, lower panel), the increase in the phosphothreonine signal at 45 min cannot be attributed to a change of ERα level and thus must represent an increase in specific Thr phosphorylation.

Identification of Thr^{311} as the p38-mediated phosphorylation site on ERα. To determine which of the ERα Thr residues is phosphorylated, in vitro-phosphorylated ERα protein was eluted from the gel, digested with trypsin, and subjected to two-dimensional separation on thin-layer chromatography (TLC) plates. The phosphopeptide mapping analysis showed the presence of two major phosphopeptides (Fig. 4a), suggesting that p38 may phosphorylate the ERα at two separate sites. Alternatively, the two spots may represent isoforms or products of partial tryptic digestion of the same phosphopeptide. The minor spots in the chromatogram are likely due to phosphorylation of the ERα protein by background kinases contaminating the p38 immunoprecipitates.

Following the tryptic mapping, each of the two major spots was recovered from the TLC plates and subjected to manual Edman degradation. As shown in Fig. 4b and c, both peptides released their ^{32}P at cycle 8, indicating that the phosphorylated amino acids are at the eighth position counting from the amino terminus of the tryptic peptides.

Next, phosphorylated ERα protein was digested with both trypsin and a secondary endoproteinase, Glu-C or Asp-N, which cuts peptides at the carboxyl terminus of glutamate and the amino terminus of aspartate, respectively. As indicated by the altered migration of the peptides after double enzyme digestion, both phosphotryptic peptides were cleaved by Glu-C (Fig. 4d) and Asp-N (data not shown). This suggests that both peptides contain Glu and Asp residues in their sequences. Manual Edman degradation of the two peptides obtained by secondary digestion with either Glu-C (Fig. 4e and 4f) or Asp-N (data not shown) released the ^{32}P at cycle 8, suggesting that the tryptic peptides contain no Asp or Glu residues at positions amino terminal to the phosphothreonine.

Based on the above analysis, the p38 phosphorylation site(s) should satisfy the following criteria. The site(s) is a Thr residue. It should be located at the eighth position from an arginine or lysine. The tryptic peptides should contain both Glu and Asp residues. The Glu and Asp residues must be located at positions carboxyl terminal but not amino terminal to the phosphothreonine. Examination of the ERα protein sequence revealed that only Thr^{311}, located in the amino terminus of the ERα ligand-binding domain, satisfies all these criteria and is therefore the p38 phosphorylation site (Fig. 4g). It becomes obvious that the two major spots generated by protease digestions both contain Thr^{311}, suggesting that they are most likely two isoforms of the same peptide.

To determine whether Thr^{311} is the major threonine phosphorylated in vivo, we mutated the site to alanine and analyzed the threonine phosphorylation of the mutant ERα (T311A). As shown in Fig. 5, the mutation eliminated the 17β-estradiol-induced ERα phosphorylation on Thr residues (Fig. 5, upper panel) but did not affect the level of ERα expression (Fig. 5, lower panel), suggesting that Thr^{311} is the major site phosphorylated in vivo. Together with the data in Fig. 3b showing that the p38 inhibitor blocks 17β-estradiol-induced ERα phosphorylation on Thr residues, the analysis shows that the p38 pathway induced by estrogens mediates Thr^{311} phosphorylation in vivo.

Regulation of ERα transcriptional activity by Thr^{311} phosphorylation. Since our previous study showed that inhibition of p38 prevented estrogen- and MEKK1-induced ERα activation (20), it was expected that mutation of Thr^{311} would decrease the ERα activity and abolish the receptor's response to MEKK1 and p38. Therefore, the transcriptional activities of wild-type ERα and the T311A mutant were compared.

As shown in the upper panel of Fig. 6a, 17β-estradiol activated the wild-type ERα about 15-fold but induced negligible activity in the mutant ERα. Since the level of ERα expression was not affected by the mutation (Fig. 6a, lower panel), this demonstrates that the mutation decreased the specific activity of the receptor. The activity of the mutant receptor was essentially undetectable unless a very high level of the expression plasmids was transfected into the cells, at which point the activity of the wild-type receptor started decreasing (Fig. 6b), presumably due to the squelching effect of excessively expressed receptors.

Further studies showed that the activity of the T311A mutant was not increased by MEKK1, whereas wild-type ERα was activated threefold by MEKK1 (Fig. 6c). In addition, SB 203580 did not decrease the activity of the T311A mutant (Fig. 6d), while the transcriptional activity of wild-type ERα induced by 17β-estradiol was decreased by 60% by the p38 inhibitor (data not shown). These studies demonstrate that Thr^{311} phosphorylation is required for ERα activation by both ligands and kinases.

Lack of effect of Thr^{311} mutation on receptor's ability to bind ligand. Since Thr^{311} is located in helix 1 of the receptor's ligand-binding domain, we first determined whether the mutation of Thr^{311} to alanine affected hormone binding. Wild-type or mutant ERα was transfected into ERα-negative Ishikawa cells, and hormone binding was determined after the cells were incubated with ^{3}H-labeled 17β-estradiol at different concentrations. As shown in Fig. 7a, wild-type and mutant receptors exhibited no difference in specific estrogen binding at both saturating and nonsaturating doses. Consistent with the lack of

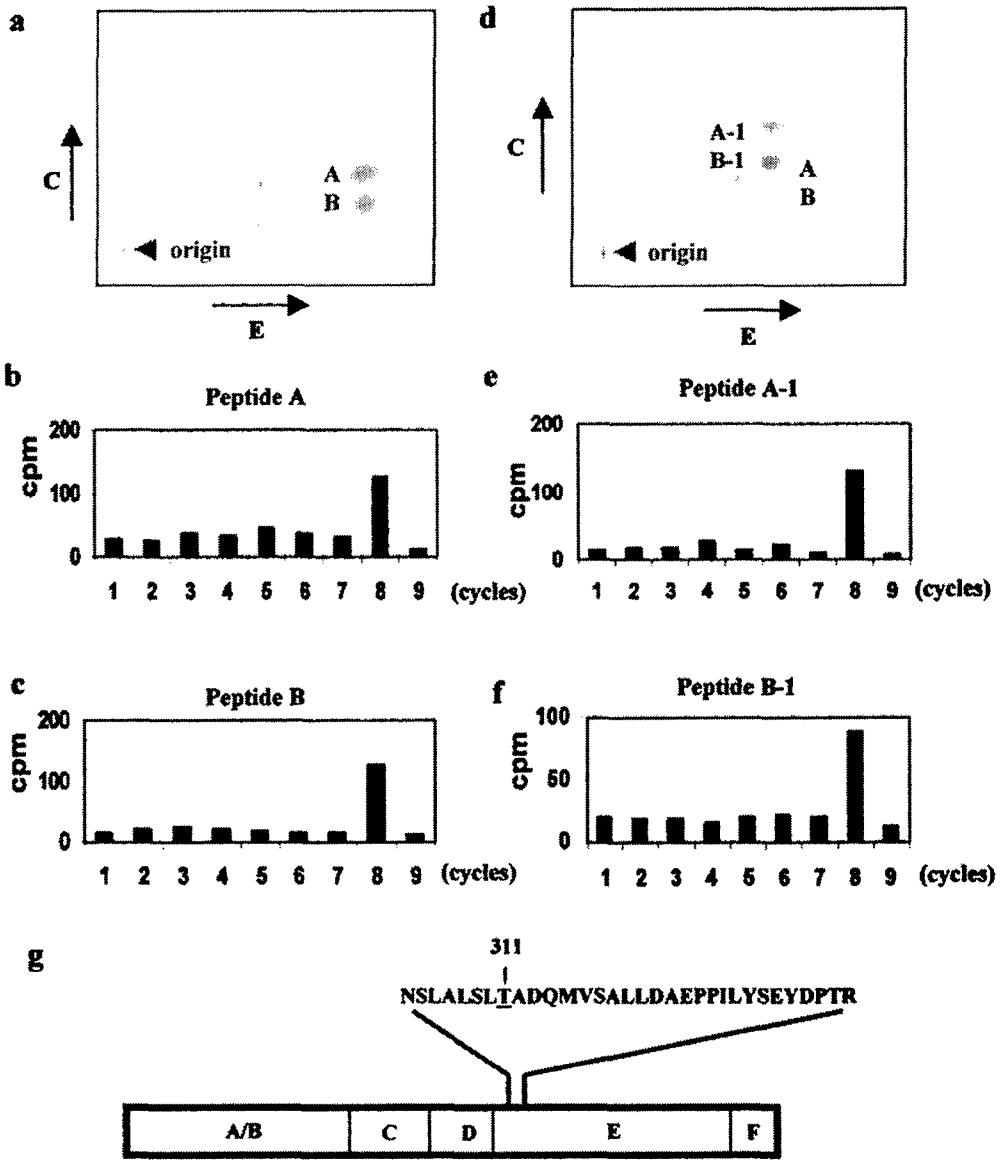

FIG. 4. Thr^{311} is the site phosphorylated by the p38 immunocomplex in vitro. (a) Phosphopeptide map of ERα after tryptic digestion. ERα phosphorylated in vitro by p38 was subjected to tryptic digestion and two-dimensional separation. The two major phosphopeptides were labeled A and B. E, electrophoresis; C, chromatography. (b and c) Edman degradation of tryptic peptides. Following autoradiography, peptides A and B were recovered from the TLC plate and subjected to manual Edman degradation. Radioactivity released from each cycle was plotted. (d) Phosphopeptide map of ERα after trypsin/Glu-C double digestion. Tryptic digests of phosphorylated ERα were cut with Glu-C and subjected to two-dimensional separation. The two major peptides were identified as A-1 and B-1. Note the presence of tryptic peptides A and B due to incomplete Glu-C digestion. (e and f) Edman degradation of phosphopeptides after double digestion. (g) Amino acid sequence of ERα tryptic peptide containing Thr^{311}.

an effect of the mutation on hormone binding activity, the decreased receptor activity caused by the mutation was not recovered by treatment with excess amounts of 17β-estradiol (Fig. 7b).

Regulation of ERα nuclear export by Thr^{311} phosphorylation. Nucleus-cytoplasm shuttling of proteins, including nuclear hormone receptors and their cofactors, has been shown to be regulated by phosphorylation (15, 18, 27, 47). In ERα, Thr^{311} appears to be located adjacent to the third nuclear localization signal (amino acids 299 to 303) (44) and within a putative nuclear export signal similar to that described for the inhibitor of protein kinase A (42) and p53 (46). The putative nuclear export signal is conserved in ERα from different species (Fig. 8a). Accordingly, it is possible that Thr^{311} phosphorylation controls the nuclear localization of ERα.

To address this possibility, ERα-negative Ishikawa cells were transfected with ERα vector and treated with SB 203580, and the nuclear-cytoplasmic distribution of the ERα was analyzed. As shown in Fig. 8b(1), poly(ADP-ribose) polymerase (PARP) and Hsp60 were clearly separated into the nuclear and cytosolic fractions under our conditions, demonstrating the efficiency of our fractionation procedure. As can be seen in Fig. 8b(2), in the absence of estrogens, ERα was evenly distributed between the nucleus and the cytoplasm, which is consistent

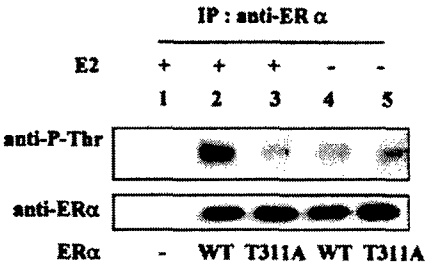

FIG. 5. Thr^{311} is phosphorylated in vivo. ERα-negative Ishikawa cells were transfected with 0.5 μg of pLEN-ERα expressing either wild-type (WT) or T311A mutant ERα, as indicated, and treated with 17β-estradiol (E2) or vehicle. The level of Thr phosphorylation was determined as for Fig. 2c. WT, wild-type ERα; T311A, ERα in which Thr^{311} was replaced with an alanine. IP, immunoprecipitation.

with our data that p38 activity is induced by 17β-estradiol. No ERα signal was detected in cells transfected with the control vector, showing the ERα-negative status of the cells as well as the specificity of the antibody for the ERα. After 17β-estradiol treatments, the receptor was located in the nucleus, as shown in Fig. 8b(3), lanes 1 and 2, whereas it remained in the cytoplasm after cotreatment with the p38 inhibitor, as shown in Fig. 8b(3), lanes 3 and 4. This demonstrates that p38 is involved in ERα nuclear localization.

The p38 MAPK may regulate the nuclear localization of ERα by either increasing nuclear import or decreasing export. In order to distinguish between these possibilities, ERα-positive Ishikawa cells were treated with leptomycin B, a known exportin inhibitor, prior to analysis of the cellular distribution of endogenous ERα. As shown in Fig. 8b(3), lanes 5 and 6, leptomycin B restored the 17β-estradiol-induced ERα nuclear localization in the presence of the p38 inhibitor, demonstrating that phosphorylation mediated through p38 inhibits Crm1-dependent ERα nuclear export. Accordingly, mutation of Thr^{311} to alanine blocked estrogen-induced nuclear localization, as shown in Fig. 8b(3), lanes 7 and 8, which was restored by leptomycin B treatment, as shown in Fig. 8b(3), lanes 9 and 10. It appears, therefore, that it is nuclear export that is controlled by Thr^{311} phosphorylation of ERα mediated through the p38 MAPK pathway.

Since the separation of nucleus and cytoplasm by fractionation is considered to determine only nuclear retention, the cellular distribution of the wild-type and T311A mutant ERα forms was analyzed by direct immunofluorescence staining. As shown in Fig. 8c, treatment with 17β-estradiol induced the nuclear distribution of the wild-type receptor but not the T311A mutant. By comparing the FITC and DAPI signals, T311A in the presence of estrogens appeared to accumulate in the areas surrounding the nuclei. The exact meaning of this

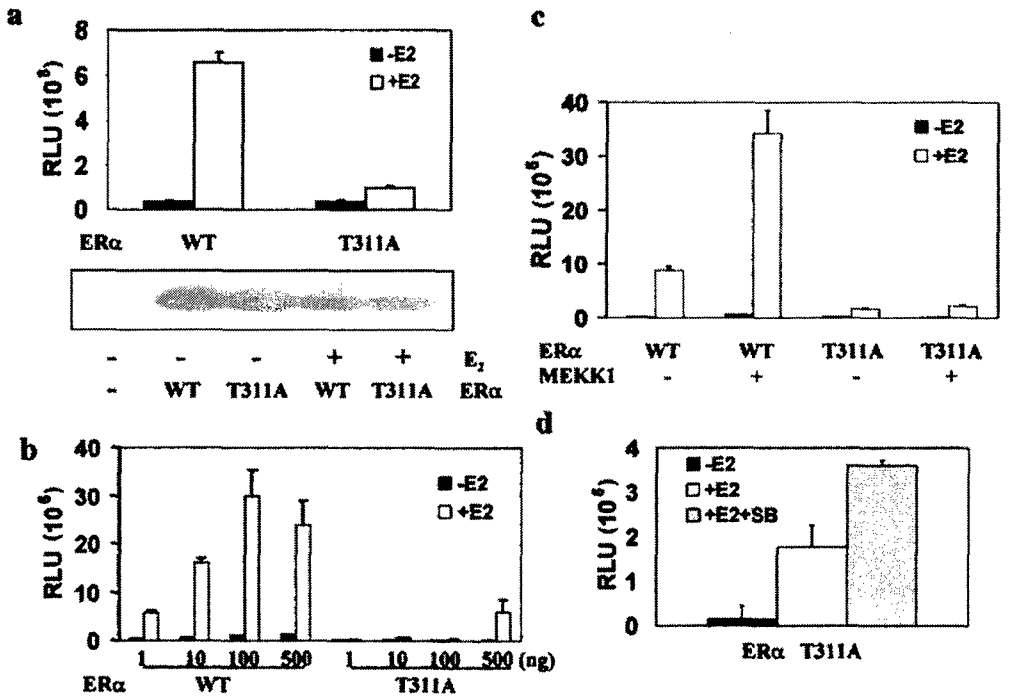

FIG. 6. Mutation of Thr^{311} to alanine decreases the transcriptional activity of ERα. (a) Lack of T311A activation by 17β-estradiol. ERα-negative Ishikawa cells were transfected with 0.1 μg of either wild-type (WT) or mutant (T311A) pLEN-hERα together with 0.5 μg of pLENβGal and 0.5 μg of EREe1bLuc and then treated overnight with 17β-estradiol (+E2) or vehicle (−E2). ERα transcriptional activity was normalized to β-galactosidase activity and is expressed as relative luciferase units (RLU) (upper panel), and the level of ERα protein was analyzed in parallel (lower panel). (b) Transcriptional activity of the T311A mutant at different dosages. Cells were transfected with 0.5 μg of EREe1bLuc, 0.1 μg of pLENβGal, and different amounts of ERα plasmid as indicated. ERα activity was determined as for panel a. (c) Lack of T311A activation by MEKK1. Cells were transfected with 0.5 μg of EREe1bLuc, 0.1 μg of pLEN-hERα, 0.5 μg of pLENβGal, and 0.2 μg of SRαMEKK1(CT). ERα activity was determined as for panel a. (d) Effect of SB 203580 on T311A. Cells were transfected with 0.5 μg of EREe1bLuc, 0.5 μg of pLENβGal, and 0.1 μg of pLEN-hERα expressing T311A and treated with 10^{-8} M 17β-estradiol and 50 μM SB 203580 or vehicle. ERα activity was determined as for panel a.

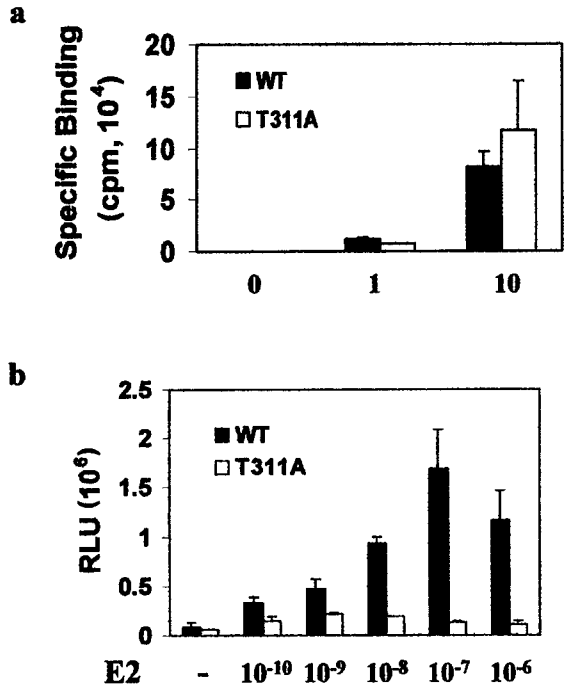

FIG. 7. Decreased transcriptional activity of T311A mutant ERα is not due to a change in hormone binding ability. (a) Similar hormone binding abilities of wild-type (WT) and T311A ERαs. ERα-negative Ishikawa cells at a density of 10^6 cells/100-mm dish were transfected with 5 μg of wild-type or T311A mutant ERα. Forty-eight hours posttransfection, in vivo hormone binding was performed as described in the text. Specific binding was calculated by subtracting nonspecific binding ([^{3}H]17β-estradiol plus a 200-fold excess of unlabeled DES) from total binding ([^{3}H]17β-estradiol alone). The data are representative of three independent experiments, and each data point was analyzed in duplicate. (b) Transcriptional activity of the T311A mutant was not restored by treatment with excessive estrogen concentrations. ERα-negative Ishikawa cells were transfected as described for Fig. 6a and treated with 17β-estradiol (E2) at the indicated concentrations. ERα activity was determined as for Fig. 6a.

observation is unclear, but it may reflect signals from the newly exported mutant ERα which have not been fully dissociated from the exporting complex. The lack of FITC signal in cells transfected with the control vector demonstrated that the FITC signal is specific to the ERα.

If the lack of transcriptional activity of T311A were due to its cytoplasmic dislocation, its restored nuclear localization would be expected to restore its transcriptional activity. The transcriptional activities of the wild-type ERα and the T311A mutant were measured in the presence and absence of leptomycin B (Fig. 9). While leptomycin B did not affect the estrogen-induced activity of wild-type ERα, the activity of the T311A mutant was increased threefold. The decreased basal activity might be due to the cytotoxicity of the drug. The lack of an effect of leptomycin B on the activity of the wild-type receptor was expected because, with respect to the effect on ERα localization, leptomycin B and ERα Thr^{311} phosphorylation should be redundant.

Regulation of ERα interaction with steroid receptor coactivators by Thr^{311} phosphorylation. Although leptomycin B selectively increased the activity of the T311A mutant, the activity of the mutant in the presence of leptomycin B was still less than that of the wild type, suggesting that the mutation affected other steps of the receptor activation process in addition to its effect on estrogen-induced nuclear targeting. Therefore, the ability of the wild-type and mutant ERαs to bind members of the p160 SRC family was compared in coimmunoprecipitation assays. As shown in the upper panel of Fig. 10, the wild-type ERα coprecipitated with both SRC1 and TIF2 in an estrogen-dependent manner, while the T311A mutant showed no binding to SRC1 and significantly reduced binding to TIF2. In parallel studies, the expression levels of the wild-type ERα and the T311A mutant were comparable (Fig. 10, lower panel), demonstrating that the decreased interaction with steroid receptor coactivators and the selective effect of Thr^{311} mutation on the interaction with SRC1 are not due to variation in the level of receptor protein expression or variation in the efficiency of the immunoprecipitations.

Regulation of biological activity of ERα in endometrial cancer cells by p38 MAPK. In endometrial cancer cells, estrogens have been shown to induce alkaline phosphatase (14). To demonstrate the significance of p38 phosphorylation on estrogen regulation of endogenous gene expression, alkaline phosphatase activity was measured in ERα-positive Ishikawa cells after estrogen treatment in the presence or absence of SB 203580. In the analysis, 17β-estradiol caused a threefold increase in alkaline phosphatase activity (Fig. 11a), which was blocked by SB 203580. The p38 inhibitor did not decrease the basal activity of alkaline phosphatase, indicating the absence of general cytotoxicity.

To further demonstrate the significance of p38 phosphorylation in the biological function of the ERα, the growth response of the ERα-positive Ishikawa cells to estrogens was assayed in the presence and absence of the p38 inhibitor. As shown in Fig. 11b, 17β-estradiol stimulated the growth of the endometrial cancer cells, which was blocked by SB 203580. Taken together with the data from Fig. 11a, these findings support the important role of the p38 MAPK as a determinant for the activation of transcriptional activity of endogenous genes through the ERα as well as its biological importance in mediating the regulation of endometrial cancer cell growth.

DISCUSSION

In summary, our studies demonstrate that the signaling pathway mediated through p38 MAKP plays an important role in ER action in endometrial cancer cells. The pathway exerts its effect by inducing ERα phosphorylation at Thr^{311}, the first threonine residue reported to be phosphorylated in ERα in response to estrogens. Although located in helix 1 of the ligand-binding domain, phosphorylation does not affect the ability of the receptor to bind the natural ligand. Instead, it blocks ERα nuclear export and promote the interaction of ERα with p160 steroid receptor coactivators, which may explain both the observed effect of the p38 MAPK pathway on the transcriptional activity of ERα in regulating endogenous gene expression as well as the biological activity of estrogens in stimulating endometrial cancer cell growth.

Although our studies clearly suggest that the p38 MAPK induced by the natural ERα agonist mediates Thr^{311} phosphorylation, it appears unlikely that p38 is the kinase that directly phosphorylates ERα at Thr^{311}. First, the Ser-Pro motif is a

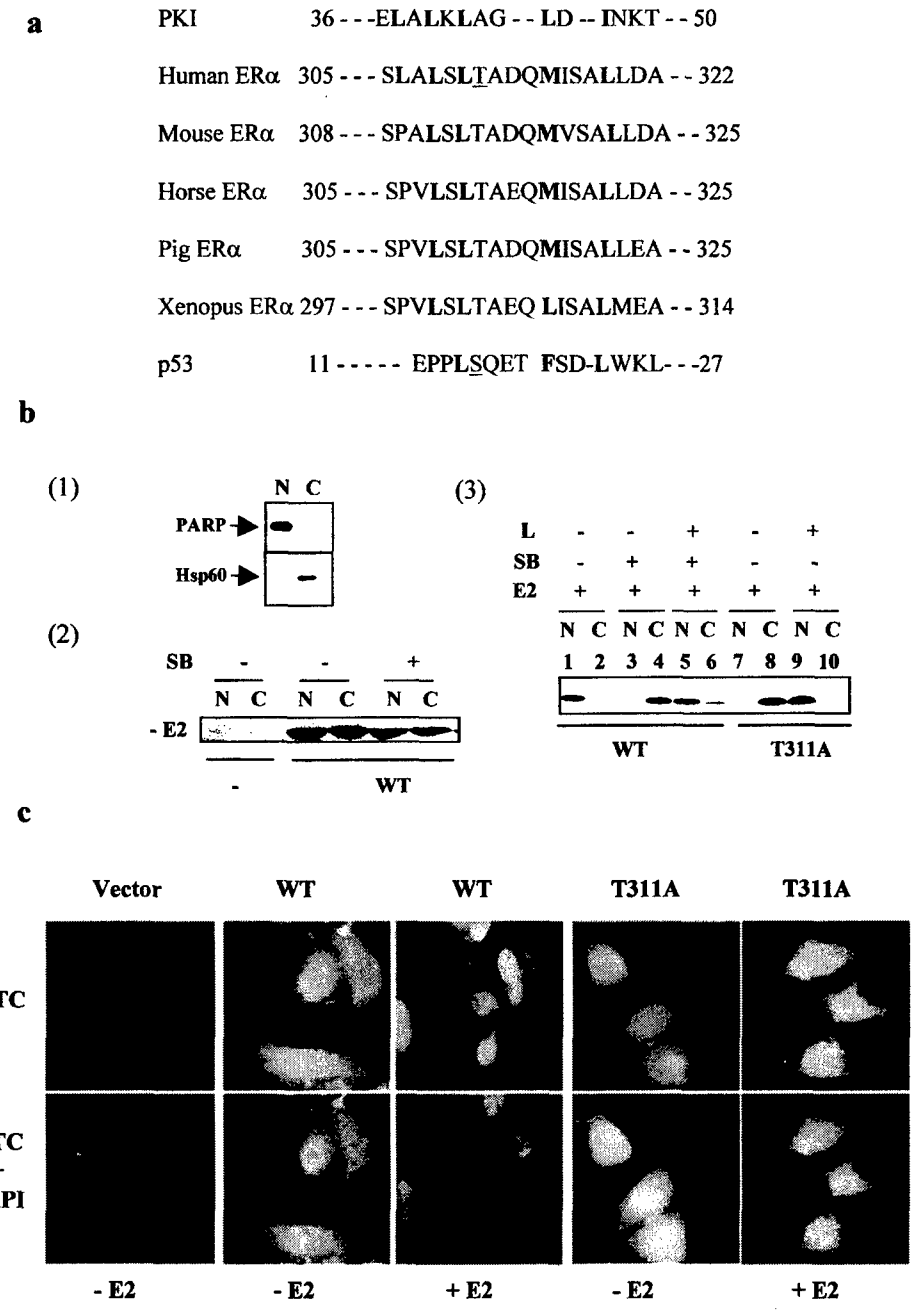

FIG. 8. Nuclear export of ERα is regulated by Thr^{311} phosphorylation. (a) The putative leucine-rich nuclear export signal sequence that is highly conserved among ERαs from different species. The amino acids critical for the nuclear export signal function of the protein kinase I (PKI) and conserved in ERα and p53 are bold. Note the close similarity between the conserved Thr^{311} and serine-15 of p53, which are both underlined. (b) Panel 1, separation of nuclear and cytosolic proteins. Nuclear (N) and cytosolic (C) extracts were separated as described in the text and confirmed by immunoblotting with antibodies against known nuclear (PARP) and cytoplasmic (Hsp60) markers. Panel 2, lack of effect of p38 inhibition on ERα nuclear localization in the absence of estrogens. ERα-negative Ishikawa cells were transfected with parental vector or pLEN-hERα and treated with vehicle or SB 203580 for 45 min. Cellular localization of ERα was determined with anti-ERα antibody. Panel 3, regulation of Crm1-dependent nuclear export of ERα by p38 MAPK-mediated Thr^{311} phosphorylation. Cells were transfected with pLEN-hERα and treated with 17β-estradiol (E2), SB 203580 (SB), leptomycin B (L, 5 ng/ml), or a combination. Leptomycin B was added to the cells 30 min before treatment with 17β-estradiol or SB 203580. (c) Visualization of subcellular distribution of wild-type (WT) and T311A mutant ERαs by direct immunofluorescence staining. ERα-negative Ishikawa cells were plated on coverslips and transfected with vector or wild-type or T311A mutant ERα DNA. Forty-eight hours posttransfection, cells were treated with 17β-estradiol for 30 min and fixed. Following immunostaining as described in the text, ER subcellular distribution (FITC signal) and cell nucleus (DAPI signal) were visualized, and images were captured under a fluorescence microscope.

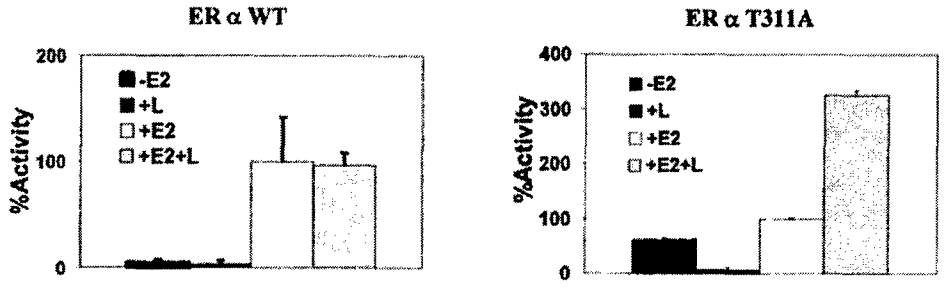

FIG. 9. Exportin inhibitor increases the activity of T311A but not the wild-type (WT) ERα. ERα-negative Ishikawa cells were transfected with 0.5 μg of EREe1bLuc, 0.5 μg of pLENβGal, and 0.1 μg of pLEN-hERα expressing wild-type or T311A ERα and treated overnight with vehicle, 17 β-estradiol (E2), leptomycin B (L), or a combination. ERα activity was expressed as a percentage of the activity in cells treated with 10^{-8} M 17β-estradiol but without leptomycin B.

feature shared by substrates for known MAPKs, and Thr^{311} is not followed by a proline. Second, SB 230580, when added directly to the kinase reactions, failed to block the in vitro phosphorylation of the recombinant ERα by the p38 immunocomplex (Fig. 2b), although the same inhibitor blocked estrogen stimulation of Thr^{311} phosphorylation in vivo and ATF-2 phosphorylation by purified human recombinant p38 kinase in vitro. Third, purified recombinant p38 did not phosphorylate ERα protein in in vitro kinase assays, whereas it phosphorylated ATF2 in parallel reactions (Fig. 2b). However, under the same conditions in which ERα phosphorylation by p38 immunocomplexes was demonstrated (Fig. 2a), large-scale affinity chromatography failed to detect any specific p38-bound protein after silver staining, even though the amount of purified p38 was readily detectable by Coomassie blue staining (data not shown). These studies suggest that the kinase phosphorylating Thr^{311} may be a member of the p38 family or a separate kinase that has a high specificity toward ERα but binds to p38 at a level too low to be detected by traditional protein-staining methods after affinity purification.

One of the main findings in these studies is that the transient ERα phosphorylation at Thr^{311} induced by the natural ligand is required for the stable localization of the ERα protein in the nucleus. The transient phosphorylation may initiate a more stable secondary event that keeps the receptor in the nucleus. For example, Thr^{311} phosphorylation may only prolong the retention of the liganded ERα in the nucleus, permitting more persistent binding of the receptor to nuclear protein or DNA components, which then obviates the requirement for phosphorylation.

From the data in Fig. 8, it appears that, in the presence of the p38 inhibitor, 17β-estradiol promotes the accumulation of ERα in the cytoplasm. It is conceivable that, in the absence of ligands, equilibrium between basal nuclear import and export determined the approximately equal distribution of ERα in the nucleus and cytoplasm in endometrial cancer cells. The binding of ligands accelerates both import and export activities, but nuclear export is blocked by the ligand-induced Thr^{311} phosphorylation. It is the suppression of nuclear export induced by ligand-activated receptor phosphorylation, instead of acceleration of import, that shifts the steady state in favor of the nuclear localization of the ERα. According to our data, the pure antagonist ICI 182,780 may target ERα to the cytoplasm because of the inability of the pure estrogen antagonist to activate the p38 MAPK.

A role of the p38 MAPK signaling pathway in promoting nuclear export of MAPK-AP kinase 2 (10) and the nuclear factor of activated T cells (NFAT) (7) has been described. On the other hand, p38 binds p53 and induces phosphorylation of serine-15 within the N-terminal nuclear export signal of p53 that blocks nuclear export (3, 46). As shown in Fig. 8, there is a close sequence homology between the leucine-rich nuclear export signal in the amino terminus of p53 and the putative nuclear export signal in the ERα containing Thr^{311}. Therefore, the effect of Thr^{311} phosphorylation on the nuclear export of the ERα is likely due to an alteration in the function of the putative nuclear export signal. However, our data do not rule out the possibility that the phosphorylation may affect nuclear export through nuclear export signal-independent mechanisms. This is particularly true because the role of the putative nuclear export signal in the intracellular traffic of ERα has not been characterized, and the existence of a functional nuclear export signal in steroid receptors is controversial (23, 41). In the study of progesterone receptor nuclear export, sequences with homology to leucine-rich nuclear export signals do not appear to be functional (41). In addition, studies by Liu et al.

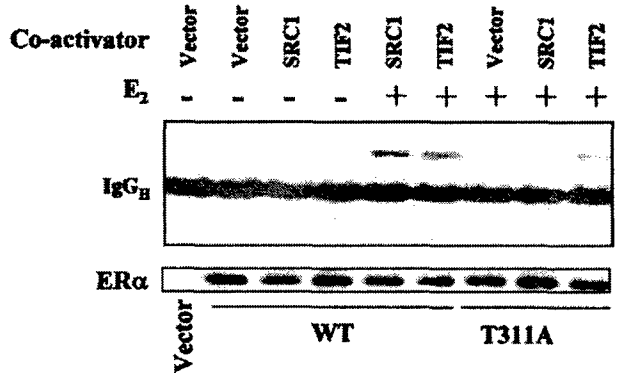

FIG. 10. Effect of T311A mutation on coactivator binding. Ishikawa cells were plated in 35-mm dishes at 3×10^5/dish and transfected with 0.5 μg of pBind-SRC-1 or pBind-TIF2 and 0.5 μg of wild-type (WT) or T311A mutant DNA. Cells were treated for 30 min with 17β-estradiol (E_2) or not, and cellular extracts from duplicate dishes were pooled and subjected to immunoprecipitation with anti-ERα antibody, followed by immunoblotting with the anti-Gal4 DNA-binding domain (upper panel) or anti-ERα (lower panel) antibody.

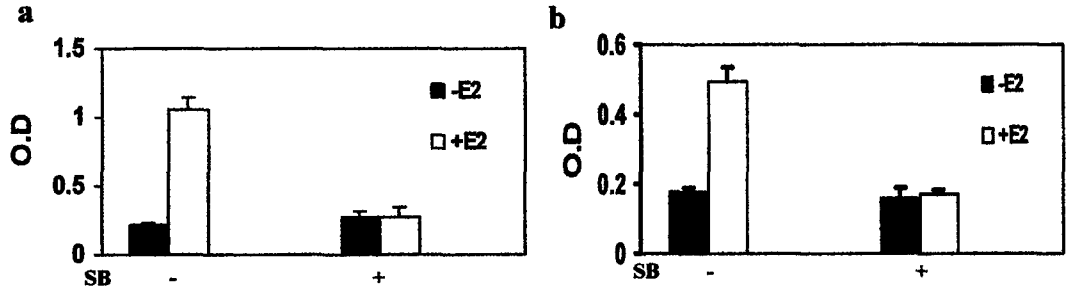

FIG. 11. p38 MAPK signaling pathway regulates the biological activity of ERα in endometrial adenocarcinoma cells. (a) Inhibition of estrogen induction of alkaline phosphatase by p38 inhibitor. ERα-positive Ishikawa cells were treated for 72 h with vehicle, 17β-estradiol (E2), SB 203580 (SB), or a combination, and alkaline phosphatase activity was measured by colorimetric enzyme-linked immunosorbent assay. (b) Inhibition of estrogen stimulation of cell growth by the p38 inhibitor. ERα-positive Ishikawa cells were treated as for panel a for 4 days, and cell growth was measured by MTT assays. O.D., optical density.

(23) showed that glucocorticoid receptors did not utilize the exportin/Crm1 pathway for nuclear export.

Besides the effect on ER nuclear localization, our studies demonstrated that Thr^{311} phosphorylation of ERα regulates the receptor's interaction with steroid receptor coactivators. More interestingly, Thr^{311} phosphorylation preferentially affected the interaction with SRC-1, which has recently been shown to be expressed at a higher level in uterine than in breast cells and may contribute to the uterine-selective agonistic activity of tamoxifen (34). Our earlier studies demonstrated that MEKK1 increases the agonistic activity and decreases the antagonistic activity of 4-hydroxytamoxifen (20). Together with the stimulation of p38 activity by 4-hydroxytamoxifen shown in Fig. 1, our data support a role of the p38 MAPK signaling pathway in determining the tissue-selective agonistic activity of tamoxifen in endometrial cancer cells.

Our study is the first to link hormone, kinase pathway, specific receptor phosphorylation site, and receptor transcriptional activity with biological function in one comprehensive investigation. The activation of p38 by estrogens is fast and transient, obviously due to the nongenomic effect of the hormone. Taking all the data together, the study provides a good example of how the genomic and nongenomic effects of estrogens work together to fulfill biological functions. Consistent with our finding that the p38 pathway is positively involved in estrogen stimulation of endometrial cancer cell growth, studies by Razandi et al. (31) showed that estrogen activation of p38 is associated with protection of endothelial cells from hypoxia-induced apoptosis. However, Zhang et al. (45) reported that activation of p38 is coupled with ER-mediated apoptosis. The different data may be explained by the possibility that estrogen-induced p38 activity regulates estrogen action in different cells through distinct mechanisms. Consistent with this idea, studies by Razandi et al. (31) suggest that the protective effect of estrogen-activated p38 on vascular cells may be mediated through Hsp27 phosphorylation by MAPK-AP2, which is obviously different from the ERα phosphorylation by the p38 MAPK pathway proposed in our studies.

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